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MECHANISM OF ACTION OF THE PRESYNAPTIC NEUROTOXIN: TETANUS TOXIN

Annual Report

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The main goal of this study has been to identify the mechanism of action of the potent neurotoxins produced by the bacteria of the Clostridial strain. We have utilized tetanus toxin as a model system and have examined its action on the inhibition of neurotransmitter release in a cloned neural cell line, PC12. Considerable information has been obtained indicating that tetanus toxin interferes with cyclic GMP metabolism in neural cells and that this process is crucial in the intoxication pathway. First, tetanus toxin infection results in inhibition of cyclic GMP accumulation in PC12 cells. The time course for the onset of the inhibition of neurosecretion and cyclic GMP increase were identical. Further, inhibitors of phosphodiesterase restore cGMP levels and neurotransmitter release in a parallel fashion. This result provides the first evidence that the effects of Clostridial infections can be reversed by pharmacological methods. These studies have been expanded to examine the effects of tetanus toxin and cGMP in a preparation of permeabilized PC12 cells. Using this system we have found that tetanus toxin alters the sensitivity of neurotransmitter release to Ca^{2+}. Further, cGMP stimulates secretion in these permeabilized cells. Future work will focus on the site of action of tetanus toxin in the cGMP metabolic pathway, particularly on phosphodiesterases in neural cells.

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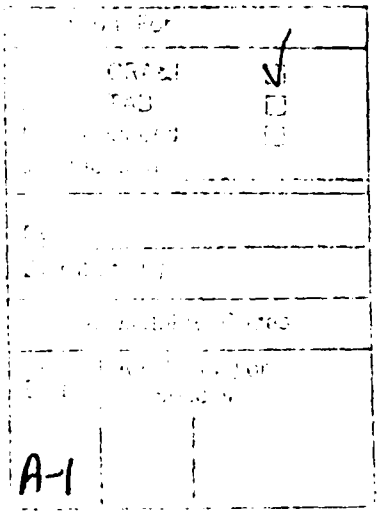
FOREWARD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on the Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)



Tetanus Toxin - Significance

Tetanus infections are no longer a serious health problem in developed countries because of effective immunization procedures. Therefore it is appropriate to ask why effort should be devoted to studying the mechanism of action of tetanus toxin. In the first place it is important to recognize that potent toxins produced by a variety of organisms have been valuable tools that have been used to probe the molecular features of the complex nervous system (Ceccarelli, and Clementi, 1979). For example, the sodium channel and the nicotinic acetylcholine receptor have been well characterized as a result of the use of tetrodotoxin and α-bungarotoxin, respectively. Therefore one important reason to study tetanus action is that it may shed light on unknown molecular processes that occur in the brain.

The chemical communication of signals between neurons across the synaptic cleft, referred to as synaptic transmission, is mediated by neurotransmitter substances and is a crucial process in the nervous system. Yet, the molecular processes that underlie the neurotransmitter release mechanism in the presynaptic cell are not understood. Accordingly, it would be extremely valuable to have toxins that could be used as tools to probe this specific process.

Tetanus toxin, a protein produced by the bacterium *Clostridium tetani*, is an extremely potent neurotoxin (Simpson, 1986; Habermann, and Dreyer, 1986). It is now well known that tetanus toxin inhibits neurotransmitter release from presynaptic terminals from a variety of neural preparations including neuromuscular junctions, primary cultured neurons, brain slices and synaptosomes (Schmitt et al.1981; Bergey et al.1983; Osborne, and Bradford, 1973). Many

laboratories have been active in trying to identify the mechanism by which tetanus brings about this inhibition. From such studies it is now clear that tetanus toxin does not: (1) cause cell death or disrupt the ultrastructure of the presynaptic terminal (Mellanby, and Green, 1981; Schwab, and Thoenen, 1976); (2) alter the synthesis, storage or uptake of neurotransmitter (Collingridge et al.1980); (3) modify presynaptic action potentials or inward calcium currents (Dreyer et al.1983). Thus the current hypothesis for tetanus toxin action is that this toxin acts by perturbing the coupling of excitation to neurotransmitter secretion at a step that occurs downstream from Ca^{2+} entry into the neuron. Tetanus toxin is one member of a small class of unique neurotoxins that act at the presynaptic terminal on processes directly involved with neurotransmitter release. All of the evidence gathered to date strongly supports the idea that tetanus toxin is indeed a very valuable tool to study excitation-secretion coupling in the central nervous system.

A second important reason to study the action of tetanus toxin is that its mechanism of action is strikingly similar to that of another potent toxin, botulinum toxin, which is produced by another closely related gram positive bacterium, *Clostridium botulinum* (Simpson, 1986). In contrast to tetanus infections, immunization and protection against botulinum infections is very limited. Thus, an understanding of the action of tetanus should yield information which will lead to a therapeutic strategy for the treatment of the toxic sequelae of the very serious botulinum infections.

Results from the Principal Investigator's Laboratory

During the Past Year

During the initial phase of this research program, considerable effort was devoted to developing cultured cells systems that could be used as appropriate models in which to investigate the mechanism of action of tetanus toxin on neurotransmitter release (Staub et al.1986; Walton et al.1988; Sandberg et al.1989). This phase of the project has been very productive as we have established that pheochromocytoma cell line, PC12, when cultured with nerve growth factor has a large concentration of high affinity tetanus toxin receptors (Walton et al.1988). Further, we have recently reported that these cells are very sensitive to the effects of tetanus toxin (Sandberg et al.1989). Detailed kinetic studies further revealed that the intoxication pathway in these cells was analogous to that which has been studied in *in vivo* systems (Sandberg et al.1989). During the past year we have continued to exploit this cell system and have identified a role for cGMP on the action of tetanus toxin. This hypothesis is based on the observations that analogues of cGMP or inhibitors of cGMP phosphodiesterase reverse the effects of tetanus toxin in PC12 cells (Sandberg et al,1989b). A major focus has been to examine the metabolism of cGMP in PC12 cells in detail.

It is well recognized that cGMP levels rise in nervous tissue in response to depolarizing stimuli (Nathanson, 1977; Goldberg, and Haddox, 1977). We have examined the effects of depolarization on cGMP levels in PC12 cells. As shown in Fig. 1, when PC12 cells were stimulated with veratridine, K^+, carbachol, or Ba^{2+}, cGMP levels were increased 7-12 fold.

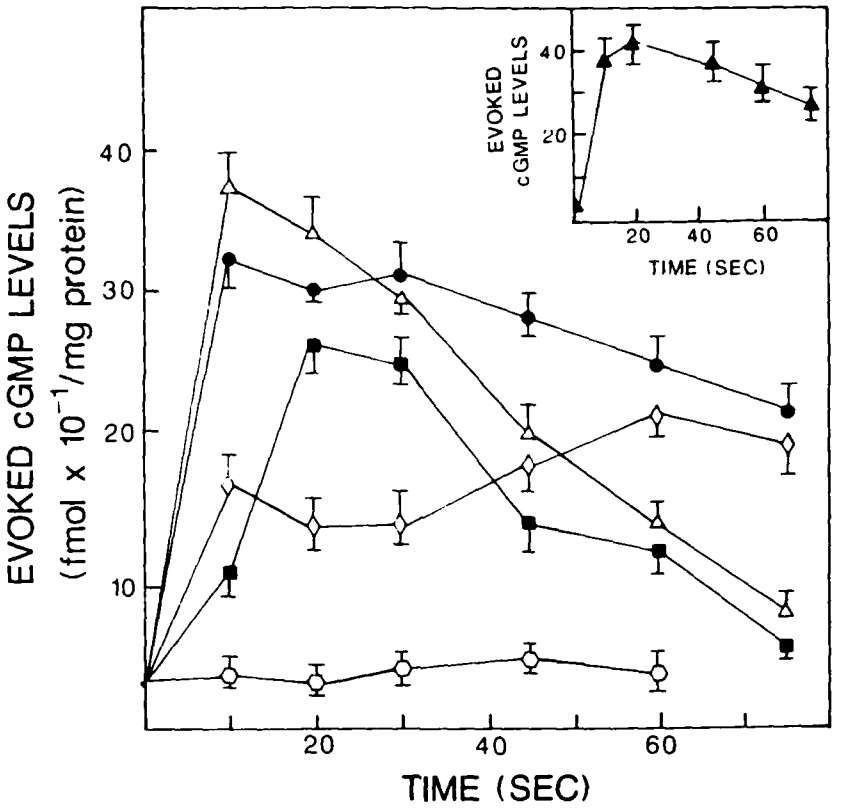


Fig. 1 Time Course of stimulus-induced cGMP accumulation in PC12 cells. Cells were cultured in 35 mm dishes with NGF. The experiments were initiated by incubating the attached cells with depolarizing buffers at 37°C. cGMP levels were measured by RIA methods. Shown are the cGMP levels when the cells were exposed to buffer supplemented with 200 μM veratridine (■), 1 mM carbachol (▲), 2 mM $Ba^{2+}Cl_2$ (◇), or 30 mM KCl (△). Inset shows the time course for cGMP levels in cultures that have been treated with carbachol in an identical manner except that PC12 cultures were pretreated for 2 min with 100 μM IBMX. These results are the means of 2-3 experiments each performed in sextuplicate (±SEM).

Time course studies revealed that there was a biphasic response, a rapid increase, followed by a declining phase. This declining phase is most likely due to the activity of phosphodiesterase since the PDE inhibitor, IBMX, attenuated this phase (Fig. 1, inset).

An important discovery was that tetanus toxin blocks the depolarization-induced increases in cGMP. As shown in Table 1, when PC 12 cells were preincubated with 10 nM tetanus toxin for 16 hr, the cGMP response to all of the depolarizing stimuli were inhibited by as much as 80 %.

TABLE I.
Effect of Tetanus Toxin on Depolarization Induced Accumulation of cGMP

Incubation Conditions	Intracellular cGMP levels (fmol x 10^{-1}/mg protein)		
	Control	Toxin	% Control
Veratridine	31 ± 1.5	12 ± 0.5	39
Carbachol	37 ± 2.7	13 ± 0.7	35
Barium	74 ± 5.0	15 ± 0.6	20
Potassium	106 ± 7.8	39 ± 3.1	37

The effects of tetanus toxin on cGMP accumulation were studied in more detail. The potency of tetanus toxin and the time course for its effects were characterized. The results are shown in Figs. 2 and 3.

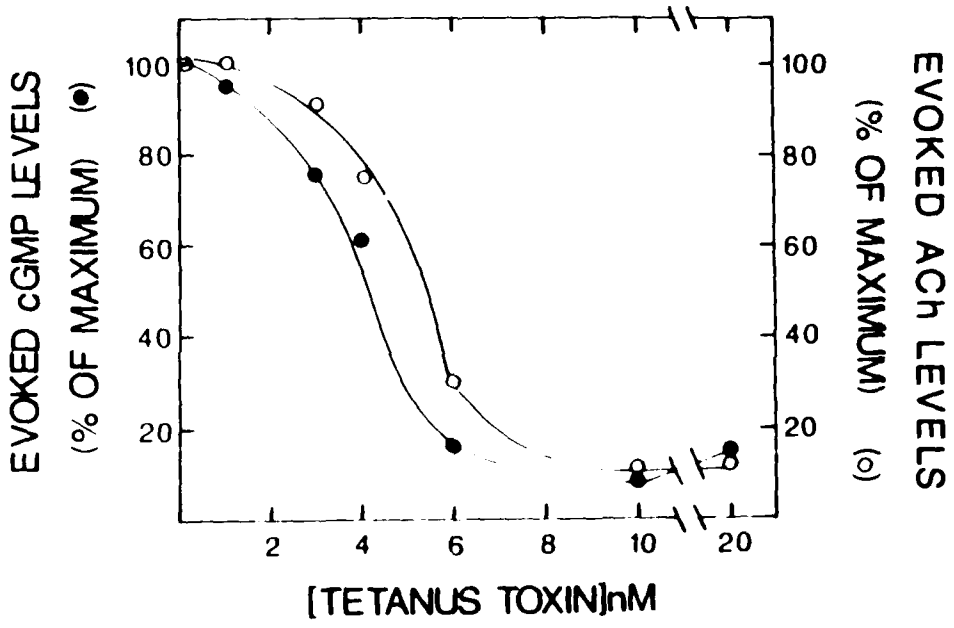


Fig. 2 *Dose-response curve of tetanus toxin action on K^+-stimulated [^{3}H]ACh release and cGMP accumulation. PC12 cells were preincubated for 3 hr with increasing doses of tetanus toxin at 37°C. At the end of the incubation period [^{3}H]ACh release and cGMP accumulation in response to stimulation with 30 mM K^+ were measured from the same culture well. Shown are the release of [^{3}H]ACh (○) and cGMP accumulation (○) after 2 min incubations expressed as percent of the maximal value in control cultures that were not exposed to toxin. These results are the means of 2-3 experiments each performed in sextuplicate.*

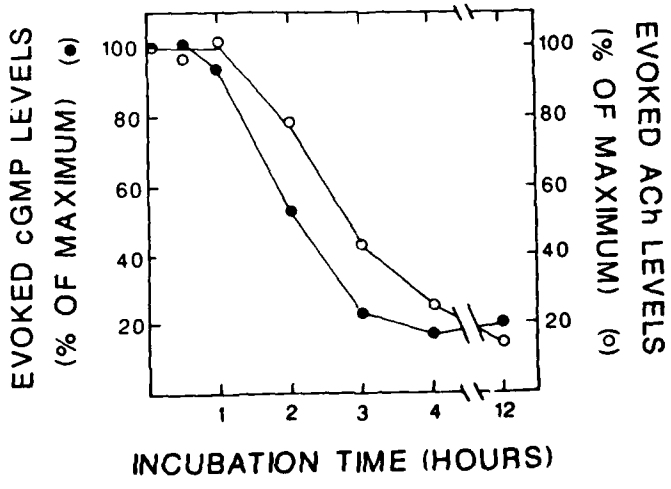


Fig. 3. *Time course of tetanus toxin action on K^+-Stimulated [^{3}H]ACh release and cGMP accumulation. [^{3}H]Ch-prelabeled PC12 cells were incubated with 10 nM tetanus toxin at 37°C. At various times the cultures were removed from the incubator and the K^+-evoked release of [^{3}H]ACh (○) and cGMP accumulation (○) accumulation were measured in the same culture wells.*

These data illustrate that there is a very close relation between the potency of toxin in inhibiting ACh release and cGMP accumulation. Further, there is a nearly identical time course for the development of the two effects evoked by the toxin. Taken together, these results provide strong circumstantial evidence that the toxin-evoked inhibition of cGMP accumulation and ACh release are causally related.

An important finding from our laboratory is that the differentiation state of the PC12 cell cultures was a crucial factor in determining the sensitivity of the cells to tetanus toxin (Sandberg et al.1989). In particular, we have found that the cells must be grown cultured in the presence of nerve growth factor (NGF) in order to obtain tetanus-sensitive cultures. Experiments were performed to determine if the tetanus toxin-evoked inhibition of cGMP accumulation was also related to cell differentiation. As shown in Fig. 4, tetanus toxin blocked Ba^{2+}-evoked cGMP accumulation only in cells that had been cultured with NGF.

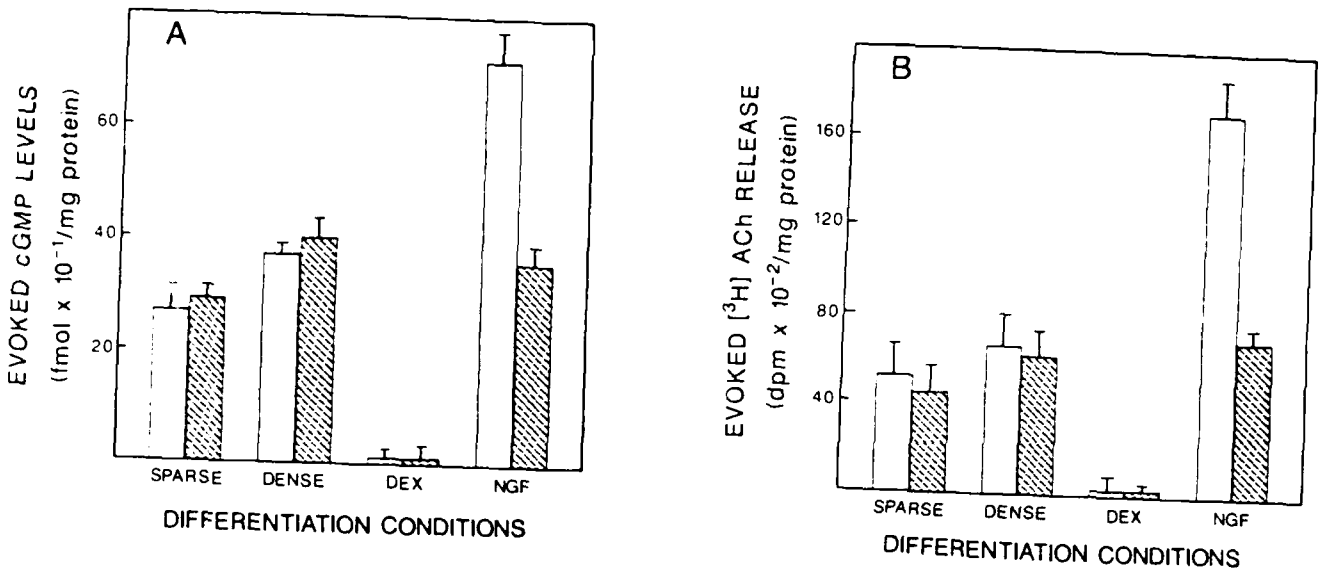


Fig. 4 Effect of tetanus toxin on Ca^{2+}-evoked [^{3}H]ACh release and cGMP accumulation from PC12 cells grown under various differentiation conditions. Ba^{2+}-evoked cGMP accumulation (Panel A) or [^{3}H]ACh release (Panel B) were measured. Evoked [^{3}H]ACh release and cGMP accumulation were measured in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM 16-18 h preincubations at 37°C) from PC12 cells grown under a variety of conditions: 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 1 x 10^{-6} M dexamethasone (DEX); 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 100 ng/ml nerve growth factor (NGF); 7 days, at high density (5 x 10^5 cells/ 10 cm^2) (Dense); or at low density (5 x 10^4 cells/ 10 cm^2) (Sparse). The results are the means of 2-3 experiments each performed in sextuplet (± SEM).

These data show that tetanus' effects on ACh release and cGMP accumulation depend on the differentiation state of PC12 cells in an identical manner. Detailed examination of the development of the toxin sensitivity in NGF-treated cultures revealed that the cells became sensitive to tetanus toxin only after culturing in NGF for at least 8 days. These results are shown in Fig. 5.

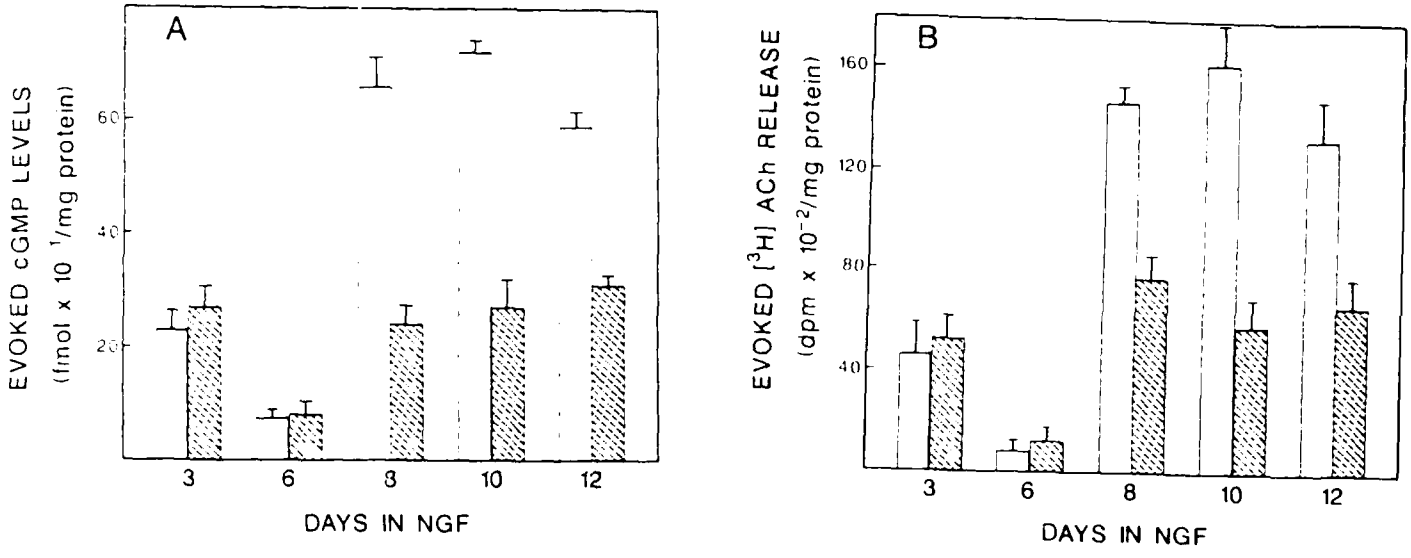


Fig. 5. Effect of tetanus toxin on Ba^{2+}-evoked [^{3}H]ACh release and cGMP accumulation from PC12 cells as a function of days in NGF. Evoked [^{3}H]ACh release (Panel B) and cGMP (Panel A) accumulation were measured as a function of culture days in NGF (100 ng/ml) in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM; 16-18 h incubation at 37°C). The results are the means of 2-3 experiments each performed in sextuplet (± SEM).

In summary, it is clear that the differentiation state of the cells is a crucial factor in determining the sensitivity of the cells to tetanus toxin as assessed either at the biochemical of functional level. The factors responsible for the expression of tetanus toxin sensitivity are intriguing but not known at present. This will be the subject of future studies.

STUDIES WITH PERMEABILIZED PC12 CELLS

During the past year considerable effort has been devoted to the

development and utilization of a permeabilized preparation of PC12 cells. The goal of this phase of the work was to use such a preparation in order to further characterize the mechanism of action of tetanus toxin. We have utilized a pore-forming exotoxin, α-toxin, obtained from *Staph. aureus*. This toxin has been utilized effectively to examine neurosecretion in several neural preparations (Ahnert-Hilger et al.1985; Thelestam, and Blomqvist, 1988). We have purified this toxin and have examined its effects on NGF-treated PC12 cells. As shown in Fig.6, this toxin is very effective in permeabilizing the cells to small ions such as Rb^+, while the cells remain relatively impermeant to larger molecules such as LDH.

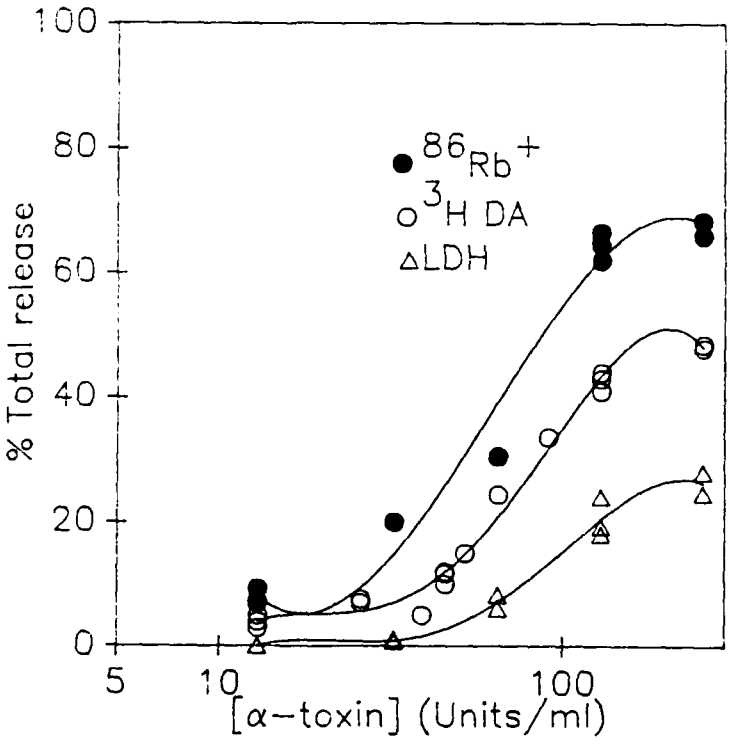


Fig. 6. Release of $^{86}Rb^+$, [^{3}H]dopamine and LDH as a function of [α-toxin). Differentiated PC12 cells were preincubated (2h/37^{0}C) with either 1.7Ci $^{86}Rb^+$/ml ($^{86}Rb^+$ efflux,●), 1.5 μCi [^{3}H] dopamine/ml (dopamine release,○) or in the absence of radioactivity (LDH release,Δ). Cells were washed in the same buffer prior to further incubation (30min/34^{0}C) with increasing concentrations of α-toxin (0-260 Units/ml). Supernatants were collected and aliquots were assayed for release of $^{86}Rb^+$, [^{3}H]dopamine or LDH. Cells were solubilized and the remaining activity measured. Values for release are expressed as % of total activity prior to

permeabilisation.

This figure also shows that it was possible to evoke dopamine release in the presence of low Ca^{2+} (20 μM) when the cells were permeabilized. In the next series of experiments the Ca^{2+}-dependency of release of DA and ACh from permeabilized PC12 cells was examined. The results are shown in Fig. 7.

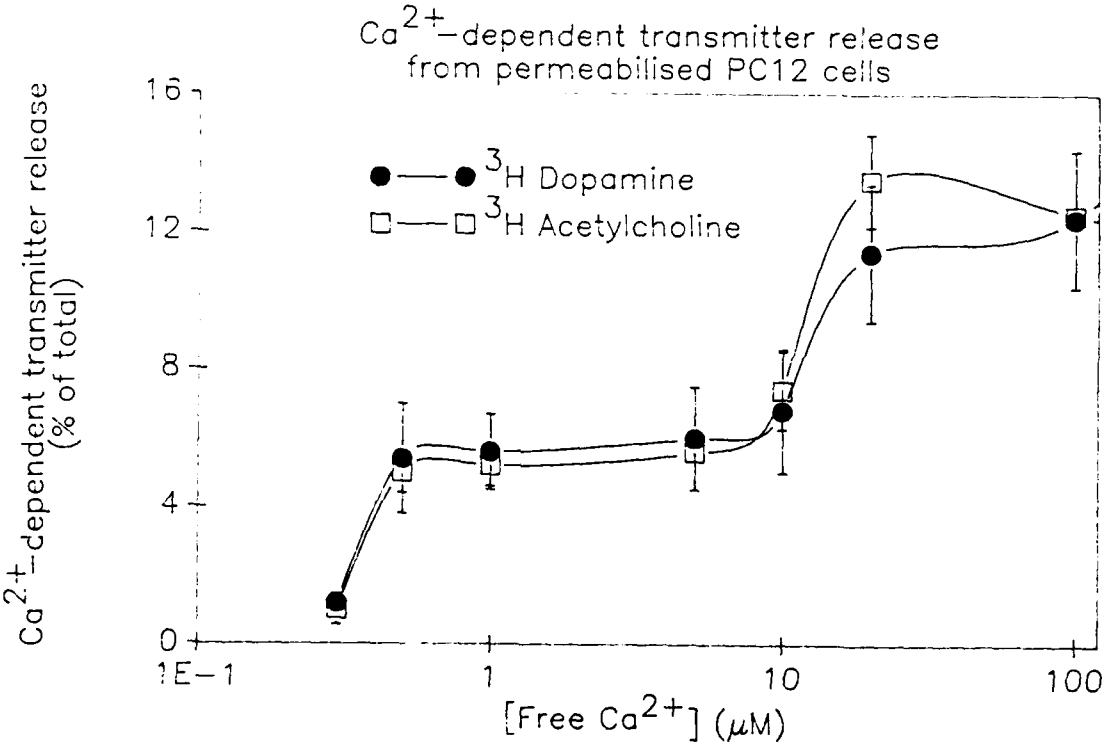


Fig. 7. Ca^{2+}-dependent release of [^{3}H]dopamine and ACh from permeabilized PC12 cells. Cells were loaded with radiolabelled transmitter and washed. Cells were permeabilized with α-toxin (30min/34^{0}C) in buffer containing the free Ca^{2+} concentrations shown. Amount of label released was assessed after centrifugation of the supernatant and is expressed as a % of the total. Values for release in the absence of added Ca^{2+} have been subtracted to yield Ca^{2+}-dependent release of [^{3}H]dopamine (○) and [^{3}H]ACh (□). Data points are averages ± SEM (n=9, DA; n=3, ACh).

There is a biphasic response to the release of both DA and ACh. The half maximal effects were observed at 0.6 μM and 20 μM free Ca^{2+}.

There is accumulating evidence in the literature that indicates that tetanus and botulinum toxins exhibit their effects by altering a step in neurosecretion that occurs downstream from Ca^{2+} entry into the neuron. This hypothesis was further tested by examining the effects of tetanus toxin on Ca^{2+}-dependent DA release from permeabilized PC12 cells. The Ca^{2+} dose response curves for DA release were determined in parallel cultures that had been preincubated in the presence or absence of 100 nM tetanus toxin. The results are shown in Fig. 8.

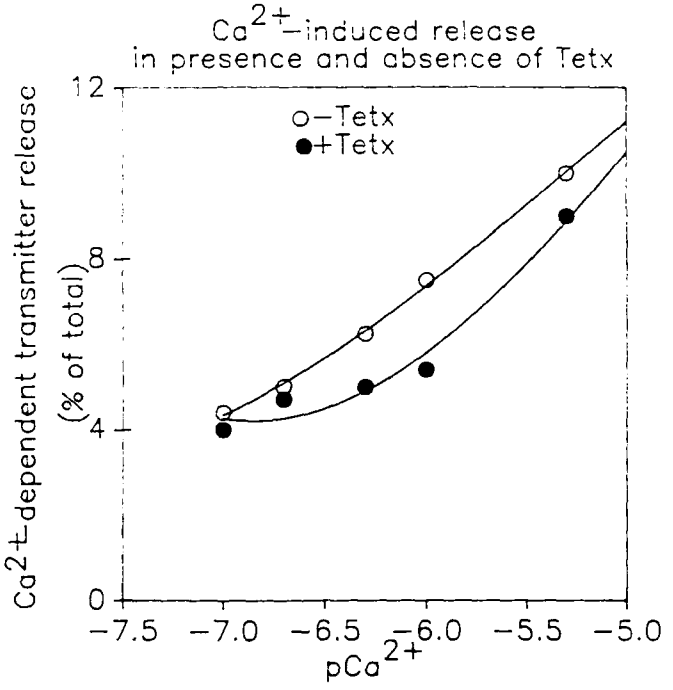


Fig. 8. Preincubation of PC12 cells with tetanus toxin; effect on the Ca^{2+}-sensitivity of the release process. Cells were washed twice in DMEM, 1% FCS prior to incubation (4h/37°C) in the same medium with (o) or without (o) 100 nM tetanus toxin. Cells were loaded with [^{3}H] dopamine, washed and permeabilized in the presence of increasing free Ca^{2+} (0-5 μM). Release in the absence of free Ca^{2+} was subtracted from all other values. Points shown are from a single representative experiment although qualitatively similar results have been obtained in two other experiments.

These results show that tetanus toxin shifts the Ca^{2+} dose-response curve to the right. That is, it lowers the sensitivity of the DA release process for Ca^{2+}. Further, these results also demonstrate that the effects of tetanus in these permeabilized cells are only seen when neurotransmitter release is evoked at low Ca^{2+}.

During several phases of this research project we have obtained evidence that cGMP is involved in the regulation of neurotransmitter release from PC12 cells. It was of interest to establish a role for cGMP in neurotransmitter release in the permeabilized cells. When permeabilized cells are incubated with cGMP in the absence of Ca^{2+} neurotransmitter release is observed (Fig. 9).

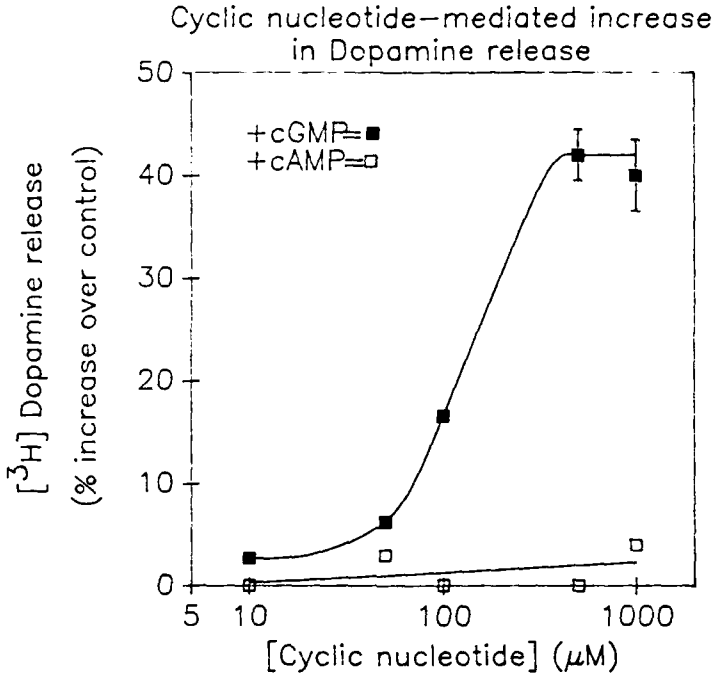


Fig. 9. Cyclic nucleotide-mediated increase in Ca^{2+}-independent transmitter release. Cells were incubated for 5min/34°C in Ca^{2+}-free medium containing cyclic nucleotide (0-1mM). Values are shown (+/- sem; n=6-12) for the % increase in release over controls (- nucleotide) mediated by cGMP (□) and cAMP (■) in Ca^{2+} free medium. Control release was 5.8-8.1% of total.

These results show that cGMP evokes DA release from permeabilized cell in the absence of Ca^{2+} with a half maximal dose of 100 μM. Further these effects are specific since cAMP, even at concentrations as high as 1 mM, fails to evoke DA release. These results are very intriguing yet the significance of these observations and the interactions of cGMP and tetanus toxin in this system remain to be identified. These will be goals of future studies.

CONCLUSIONS

During the early phase of this project we were successful in establishing a cultured cell model system, the PC12 pheochromocytoma cell line, to study the mechanism of action of tetanus toxin. We have established that these cells contain a high density of high affinity tetanus toxin receptors and are sensitive to intoxication by exposure to low doses of tetanus toxin (Sandberg et al.1989; Walton et al.1988). Further we have studied the characteristics of the intoxication pathway (Sandberg et al.1989) and have found that it is analogous to that which has been characterized, to some extent, in vivo (Simpson, 1986; Habermann, and Dreyer, 1986). The major thrust during the past year was to exploit this well characterized model system to gain insight into the molecular mechanism of action of tetanus toxin.

Our results demonstrate that intracellular cGMP levels increase when PC12 cells are depolarized. The time courses for cGMP accumulation in response to various stimuli were similar. The evoked levels of cGMP peaked within 20-40 sec which is consistent with the time course of neurotransmitter release under these conditions (Sandberg et al.1989). Further support for a link between function and cGMP levels was provided by the observation that there was a proportional relation between evoked cGMP levels and ACh release under a variety of conditions of depolarization and cell growth. It has been recognized for many years that cGMP levels in neural tissues increase in response to depolarizing stimuli (Nathanson, 1977; Goldberg, and Haddox, 1977). However the functional significance of this effect has not been elucidated. Our studies with a homogeneous cell line provide strong circumstantial evidence for a role of this cyclic nucleotide in neurosecretion.

The relationship between cGMP accumulation and neurotransmitter release was further underscored by the observation that tetanus toxin inhibited both processes. There is a remarkable correlation between the toxin dose-inhibition curves and the time course for the development of the inhibitory responses of cGMP accumulation and ACh release in PC12 cells. We have previously reported that only NGF-treated PC12 cells were sensitive to tetanus toxin. The same relation was found for the toxin inhibition of evoked-cGMP accumulation . Previously, the molecular mechanisms of tetanus toxin have remained elusive. Taken together these results provide the first biochemical evidence for the underlying mechanism of action for this toxin.

Another major advancement during the past year has been the development and utilization of a permeabilized PC12 preparation. Permeabilized cells allow for control of the intracellular environment by direct application of a variety of agents into this compartment. The use of α-toxin as the permeabilizing agent has a number of significant advantages over the use of detergents, including minimal damage to the intracellular organelles. The fact that a large Ca^{2+}-dependent release of neurotransmitter was observed in permeabilized cells supports this conclusion.

The permeabilized preparation has been exploited to examine the effects of tetanus toxin on the role of Ca^{2+} in neurosecretion. The results reported here support the hypothesis that part of the action of tetanus toxin is due to a lowering of the Ca^{2+} sensitivity of the release process. If this is true, then it should be possible to override the effects of toxin by applying high levels of Ca^{2+}. This was in fact observed. These results are consistent with observations made in studies with botulinum toxin at the neuromuscular junction (Simpson, 1986). The precise mechanism whereby tetanus brings about the reduced

sensitivity to Ca^{2+} remains to be identified. The permeabilized cell preparation represents a valuable system in which to examine this problem.

cGMP was also found to play a role in secretion in permeabilized cells. Novel results reported here indicate that cGMP evokes neurotransmitter secretion in a Ca^{2+} independent manner in permeabilized cells. This is in contrast to the effects of this nucleotide on ACh release in intact cells, where it did not stimulate neurotransmitter release by itself or upon depolarization (Sandberg et al. 1989). The relation between this phenomenon and tetanus action is not known at this time but will be a focus of future studies.

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PERSONNEL INVOLVED IN CONTRACT WORK

1. Terry B. Rogers, PhD -- Principal Investigator, 25% time
2. David Evans, PhD -- Research Associate, 100% time
3. Andrea Grandin, MS -- Research Assistant, 100% time

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